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TRANSACTIONS

Studies on Vitamin B_2 Complex. VI

Rat-acrodynia and Fatty Acids.*

By Ume Tange.

(The Institute of Physical and Chemical Research.)

Received Nov. 13, 1939.

In 1932, I reported that when young rats were maintained on the diets totally deprived of fats they developed characteristic symptoms accompanying impairment of growth, denuded area of skin, and "scaly" condition of feet, which were curable by the administration of either linoleic or linolenic acid.[1] Later, in the studies on vitamin B_2 deficiencies[2][3], it was found that rats suffering from lack of vitamin B_6 often developed symptoms similar to those of the fat deficiency mentioned above. However, when the diets contained moderate amounts of fats, for instance 10%, the symptoms were irregular and not so severe as with fat-free diets. Birch and György[4] reported that certain fats had a sparing action on vitamin B_6, and they suggested that this action was due to the linoleic acid present in the fat. Salmon[5] showed that oils alone or starch alone failed to cure or prevent acrodynia and that oils did not contain the entire dermatitis-preventing factor, but might contain an essential part of the factor which supplemented the heated yeast extract. More recently Birch[6] reported that two factors were concerned in the cure of the acrodynia-like dermatitis. One was the water-soluble factor vitamin B_6; the other was fat-soluble and present in the fatty acid fraction of maize oil, which appeared to be similar to the "linoleic acid" of Burr and Burr.

The experiments presented in this paper are, therefore, concerned with the relation between vitamin B_6 and unsaturated fatty acids in the cure and production of acrodynia-like dermatitis of rats.

Experimental.

Methods.

In order to carry out this experiment, it was needed to prepare pure casein and vitamin B_6 extract free from fats. The following procedure, therefore, was adopted.

Purification of casein.— 2 kg of casein was stirred into 5 litres of water solution of 60 g of NaCl containing 6 cc of glacial acetic acid. After settling for several hours, the supernatant liquid was decanted off, and a similar treatment was repeated six times more. This was filtered on a large Buchner's funnel, washed free from acid, and then the casein was stirred into 4 litres of 95% alcohol. The alcohol was removed by filtration. This procedure was repeated once more, the casein was dried at about 50°C, and ground, then extracted with ether for 10 days to remove fatty materials completely.

Preparation of yeast extract.— 200 g of dried brewer's yeast was extracted with 800 cc of 75% alcohol by shaking for 2 hours at room temperature. It was filtered and reextracted as above, the process being repeated twice more. The combined extracts were then evaporated down to remove the alcohol, adjusted to pH 2 with HCl, and shaken with ether several times to remove all neutral fats and fatty acids. The solution was made to about pH 6 with NaOH, and concentrated in vacuo. 95% alcohol was added into the concentrate and the mixture was allowed to stand in the ice box until the inert materials had settled out, which were filtered off and the filtrate kept for assay (10 drops of this solution correspond to the yield from 0.8 g of the dried yeast).

A. Estimation of vitamin B_6 activity of the yeast extract. The basal diet used in this experiment had the following composition:

Diet I.

Purified casein	18%
Sucrose (*Pharmacopeia Japonica*)	67
Butter fat	9
McCollum's salt mixture	4
Agar-agar	2

This was supplemented with 10 γ B_1 hydrochloride, 20 γ riboflavin each rat daily, and 2 drops of biosterin** (45.000 I. U.) weekly as vitamins A and D.

The feeding technique employed in this work was for the most part similar to that described in the preceding paper.[7]

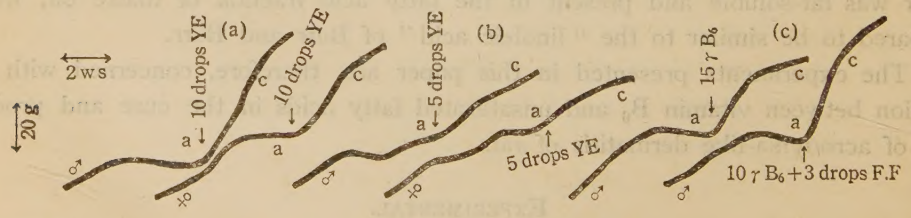

Fig. 1. Growth curves of rats on Diet I, supplemented daily by 5 or 10 drops of yeast extract, and B_6 alone or combination of B_6 and FF (filtrate factor), respectively.
a acrodynia, c cured, FF (one drop=1.5 g of fresh beef liver).

From Fig. 1 (a and b), it may be noticed that the cure of acrodynia and

the response of growth were slow with 5 drops of the yeast extract, while more rapid cure and growth resulted with 10 drops of the extract. With 15 γ of crystalline B_6,*** nearly the same result as with 10 drops of the yeast extract was obtained. By supplementing vitamin B_6 with 3 drops of filtrate factor, however, a very striking effect on growth was brought about as seen from Fig. 1 (c). This was in agreement with the previous experiments.[2]

Filtrate factor used throughout the experiments was prepared from beef liver by the method previously described.[8]

B. In consideration of the results obtained in the previous experiments[1][3], the following attempts were made; to study firstly, the influence of vitamin B_6 on the production of the fat-deficient disease, and secondly, the influence of fats in vitamin B_6-free diets on the development of the acrodynia-like dermatitis. The basal diets used in these investigations are shown in Table I.

1. Experiments on fat-free diet. This was carried out by feeding rats on diets provided with different amounts of vitamin B_6. The basal fat-free diet is given in Table I.

TABLE I.

Composition of diets (per cent.)

Component \ Diet	Diet II	Diet III	Diet IV
Purified casein	20	20	20
Sucrose (*Pharmacopeia Japonica*)	73	70	70
McCollum's salt mixture	5	5	5
Agar-agar	2	2	2
Soy bean oil	—	3	—
Crisco	—	—	3

All the above diets were supplemented as in Diet I.

Group 1. The rats receiving 10 drops of the yeast extract grew well for 5 to 7 weeks, then the growth was retarded. At about 9 to 10 weeks the nose and mouth were inflamed, and scaly feet, dandruff and alopecia appeared, but no typical acrodynia was noticed. When this condition condinued the rapid loss in weight occurred and death happened shortly unless fatty acid was fed. Administration daily of 5 to 10 drops of soy bean oil or 2 drops of linoleic acid brought about increase in weight and rapid cure, but crisco had apparently no such curing properties (Fig. 2).

Linoleic acid was prepared from linol-hydroxamic acid† $\left(C_{17}H_{31}-C\begin{matrix}NHO\\OH\end{matrix}\right)-$. 10 g of pure linol-hydroxamic acid (mp 41~42°C), which was separated from

cotton-seed oil, was added into a mixture of 100 g of 70% ethyl alcohol and 6 g of H_2SO_4, and it was heated on a water bath under reflex condenser in the atmosphere of CO_2 until no more purple red colour reaction with $FeCl_3$ appeared; it took about three hours. The resulting solution was distilled in a reduced pressure to remove the alcohol, adding a small amount of water from time to time. The solution was now extracted with petroleum ether below bp 50°C, the extraction being repeated twice more. After removing the ether by distillation, the residual solution was saponified with alcoholic potassium hydroxide in order to remove the ethyl ester which might be present. This alkaline solution was now acidified with HCl, and again extracted with petroleum ether. After dehydrating with anhydrous Na_2SO_4, the petroleum ether solution was evaporated as completely as possible in a high vacuum in CO_2 atmosphere. The yield of linoleic acid was nearly theoretical.

Group 2. The animals given 5 drops of the yeast extract grew for the first few weeks, but gradually declined in weight. They developed acrodynia-like dermatitis within 5 to 7 weeks, accompanying scurfy coat and denuded area on the skin. With 5 drops of soy bean oil, the improvement in weight and dermatitis was much slower than with 10 drops of the oil. Feeding 2 drops of linoleic acid brought about a prompt cure of dermatitis, whereas crisco was ineffective (Fig. 3).

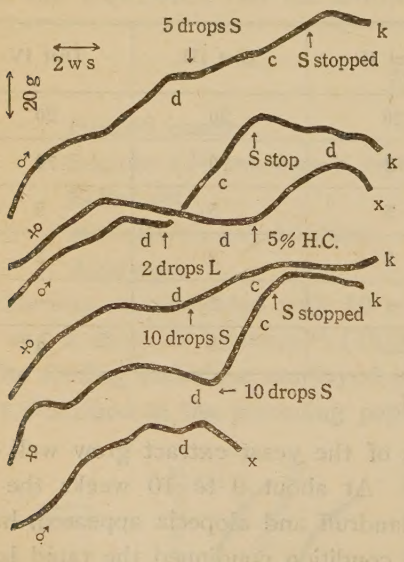

Fig. 2. Growth curves of rats on fat-free diet (Diet II), supplemented daily by 10 drops of yeast extract.

d; fat-deficiency, S; soy bean oil, L; linoleic acid, H. C.; crisco, k; killed, c; cured, x; died.

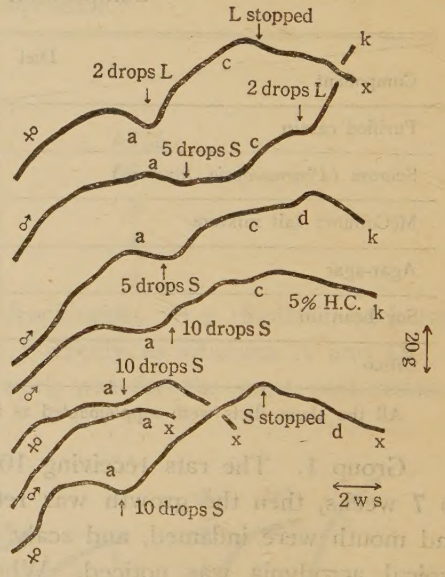

Fig. 3. Growth curves of rats on fat-free diet (Diet II), supplemented daily by 5 drops of yeast extract.

a; acrodynia, d; fat-deficiency. The other abbreviations are the same as in Fig. 2.

These results indicate that rats may develop the acrodynia-like dermatitis if diet is free from fat even when moderately large amounts of vitamin B_6 are given. It would seem, therefore, that certain fats are necessary for normal growth of rats.

2. Experiments on vitamin B_6-free diets. This was carried out by feeding animals on diets containing varying amounts of fats to determine the time of the development of acrodynia-like drematitis and the degree of the symptom. The diets used are shown in Table I.

Group 1. The rats fed on Diet II (fat-free diet) developed severe acrodynia in 3 to 4 weeks. Administration of 10 drops of the yeast extract caused some improvement on the symptom, but the rats declined in weight and death occurred among them. By the additional supplement, however, of 10 drops of soy bean oil or 2 drops of linoleic acid, there was an immediate resumption of growth and the symptoms cleared up within a few weeks. Cured animals maintained themselves free of all symptoms as long as the fatty acid and yeast extract were continued. If the oil or fatty acid was withheld, the rats declined in weight and developed acrodynia (Fig. 4).

Group 2. The acrodynia-like dermatitis appeared in about 6 to 7 weeks in the rats fed with Diet III (3% soy bean oil). By providing 5 or 10 drops of the yeast extract the acrodynia was quickly cured and growth restored. When 15 γ of B_6 was given the dermatitis was cured, but filtrate factor was needed to induce optimum growth in the rats. Soy bean oil alone did not prevent the acrodynia but did delay the onset of the symptom to some extent (Fig. 5).

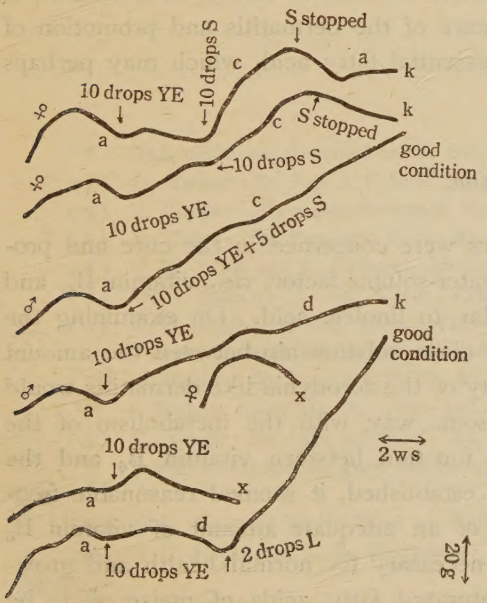

Fig. 4. Growth curves of rats on vitamin P_6-free diet containing no fat. YE; yeast extract. The other abbreviations are the same as in Figs. 2 and 3.

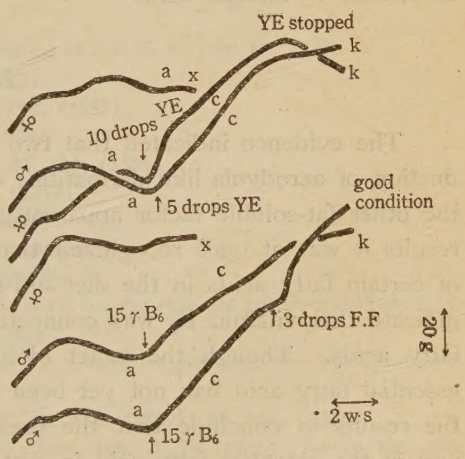

Fig. 5. Growth curves of rats on vitamin P_6-free diet containing 3% soy bean oil. YF; filtrate factor. The other abbreviations are the same as in Figs. 3 and 4.

Group 3. Results obtained with Diet IV (3% crisco) were similar to those with Diet II (fat-free) except the delayed onset of the dermatitis, which was not

so severe as seen in the animals on Diet II. Without supplement of the yeast extract, the animals died with rapid loss of weight. 5 drops of the yeast extract failed to cure the dermatitis, but 10 drops of the extract gave a slow improvement. Complete cure and normal growth were obtained by an additional supplement of either 5 drops of soy bean oil or 2 drops of linoleic acid (Fig. 6).

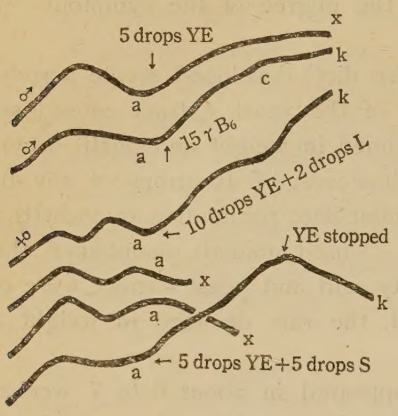

Fig. 6. Growth curves of rats on vitamin B_6-free diet containing 3% crisco.
The abbreviations are the same as in Figs. 3 and 4.

The results with 10% crisco diet were not shown in the figures but were similar to those observed with the 3% crisco diet. The development of acrodynia was irregular, and the onset was delayed, usually requiring 10 to 12 weeks.

These results show that when rats are fed on the basal fat-free diet without any addition of vitamin B_6, severe acrodynia develops very quickly, with subnormal weight and scaliness of hind feet. Feeding the yeast extract relieves the symptoms to some extent, but for copmlete cure and normal growth it is necessary also to administer certain fatty acids. It is seen that the effect of soy bean oil on cure of the dermatitis and promotion of the growth depends on its content of the essential fatty acid, which may perhaps be similar to linoleic acid.

Discussion.

The evidence indicated that two factors were concerned in the cure and production of acrodynia like dermatitis; one water-soluble factor, *viz.*, vitamin B_6, and the other fat-soluble factor apparently similar to linoleic acid. On examining the results it was at once recognized that the close relationship between the amount of certain fatty acids in the diet and severity of the acrodynia-like dermatitis would indicate that vitamin B_6 was connected in some way with the metabolism of the fatty acids. Though the exact biological function between vitamin B_6 and the essential fatty acid has not yet been fully established, it seemed reasonable from the results to conclude that the presence of an adequate amount of vitamin B_6 and of the essential fatty acid in diet was necessary for normal health and growth of the animal. Birch[6] found the unsaturated fatty acids of maize oil to be effective in relieving the symptoms of vitamin B_6 deficiency, and suggested this finding to be related to the observations of Burr and Burr[9] concerning essential fatty acids, and perhaps also to the fat-soluble antidermatitis factor indicated by Hogan and Richardson.[10] He could not findany evidence to indicate that the vitamin might exist in combination with lipoids, but he presented, instead, the evidence showing that there was a functional relationship between the unsaturated

fatty acids and the vitamin. Halliday[11] reported further evidence supporting this view, who observed that there was fatty liver in vitamin B_6-deficient animals and feeding choline remedied such condition to a large extent. Quackenbush and Steenbock[22] found that a B_6-deficient diet supplemented by unsaturated fatty acids, either as natural oils or as 10 mg per day of ethyl linoleate, protected rats from acrodynia and kept them in good health. Salmon[5] also observed a relation between B_6 and fat metabolism. Such reports led to the conclusion that vitamin B_6 was connected in some way to fat metabolism.

Summary.

Data are submitted which show that two factors are concerned in the production and cure of the acrodynia-like dermatitis. One is water-soluble factor, *viz.*, vitamin B_6; the other is fatty acid factor similar to linoleic acid.

The evidence suggests that vitamin B_6 is connected in some way with the metabolism of the fatty acids.

I wish to thank Professor U. Suzuki for his many helpful suggestions concerning this work. I am also indebted to Misses M. Takahashi and H. Sasaki for their generous assistance in preparing the materials and feeding the animals.

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** I desire to thank Mr. Kasama for supplying biosterin.

(7) U. Tange: Sc. Pap. I. P. C. R., **36**, 471, (1939).

*** I Wish to thank Mr. A. Ichiba and Miss K. Michi for the generous gift of crystalline B_6.

(8) U. Tange: Sc. Pap. I. P. C. R., **35**, 47, (1938).

† I wish to express my great indebtedness to Dr. Y. Inouye for providing the linol-hydroxamic acid.

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ABSTRACTS

from

TRANSACTIONS published in JAPANESE

(Pages refer to the Japanese originals of this volume unless otherwise noticed)

A Brown Forest Soil in Kokuga of North-Manchuria.

(pp. 125~128)

A. KAWASHIMA and G. SUYAMA

(Agr. Chem. Laboratory, Kyushu Imp. University; Received Jan. 20, 1940.)

Kokuga is situated on the river Amur in lat. 50° 15′ N. and long. 127° 29′ E. The soil profile now concerned exibits clear morphorogical characteristics of a slightly podzolized brown forest soil influenced by soil water to some extent. The data described below are all expressed on air-dry basis.

Some analytical data on fine soil are given in Table I. The exchange capacity and exchangeable calcium are expressed as mg. eq. per 100 g. soil.

Table I. Some analytical data on fine soil.

Layer	Moisture %	Loss on ignition %	Total N %	pH		Daikuhara acidity ($y_1 \times 3$)	Hydrolytic acidity (y_1)	Exchange capacity	Exchangeable Ca	% of Ca
				H_2O	KCl					
A_1	7.34	10.85	0.36	5.33	4.27	3.9	32.1	36.59	16.43	44.9
A_2	6.11	6.07	0.10	5.59	4.24	5.7	21.8	27.70	13.66	49.3
B_1	6.98	4.94	0.09	5.66	4.46	3.6	15.8	28.72	14.79	51.5

As may be seen in Table I. in A_1-layer total nitrogen content and exchange capacity are very high and the pH-value and percentage saturation of calcium are relatively low.

The colloidal clays (<0.001 mm ϕ) were separated and analysed. The total contents of silica and sesquioxides and their molecular ratios are given in Table II, in which the loss on ignition and exchange capacity are also included.

Table II. Some analytical data on colloidal clay.

Layer	Moisture %	Loss on ignition %	Exchange capacity (m. eq.)	SiO_2 %	Al_2O_3 %	Fe_2O_3 %	$\frac{SiO_2}{Al_2O_3}$	$\frac{SiO_2}{R_2O_3}$	$\frac{Fe_2O_3}{Al_2O_3}$
A_1	4.73	23.28	86.68	37.87	17.15	7.00	3.74	2.97	0.26
A_2	9.92	12.24	72.85	42.20	20.23	8.44	3.54	2.79	0.27
B_1	6.10	13.22	70.99	44.36	21.52	8.25	3.49	2.81	0.25

The high loss on ignition and exchange capacity in A_1 are due to the presence of some humus. The silica-alumina and silica-sesquioxide ratios in A_1 are the greatest, and that means some leaching down of colloidal sesquioxides from this layer. But as the differences in the magnitude of these ratios between each layer are very insignificant, a fairly good similarity of composition between these colloidal clays can be assumed.

Phosphoric Acid Absorbtion of Soils in Tyosen. (VI~VII)

(pp. 129~144)

By Misu-Hideo.

(Agricultural Experiment Station, Government General of Tyosen;

Received Aug. 28, 1939.)

On the Enzymic Action of Nucleotid-like Substances. (II)

(pp. 145~146)

By Tetsutarō Tadokoro & Tsuneyuki Saito.

(Hokkaido Imperial University; Received Dec. 26, 1939.)

On the Hydrolysis of Fats and Fatty Acid Esters. (VI)

(pp. 147~158)

By Toyoki Ono.

(Chemical Laboratory of the Fish Meal Association of Japan; Received Jan. 23, 1940.)

(I). Hydrolysis of Triglycerides by Ricinus Lipase.

Triglycerides are less attacked by ricinus lipase than by pancreas lipase, especially the hydrolysis of triricinolein took place with the smallest velocity. On the contrary, the glycerides consisting of the same ricinoleic acid, castor oil, is split rapidly.

These facts seemed to be due to the difference in the emulsification of the substrates.

(II). Hydrolysis of Esters by Pancreas and Ricinus Lipase.

(A). Forty-six esters of organic acids were prepared in this laboratory by Haller's method with acids (aliphatic and aromatic acids) and alcohols (methyl, normal and iso-propyl, normal and iso-butyl, amyl alcohols).

(B). The increase of the number of carbon atoms in alkyl group decreases

the rate of the hydrolysis of esters, and methyl esters of fatty acids are hydrolysed more easily than alkyl esters.

(C) The hydrolysis of esters has no such relation to the number of carbon atoms in fatty acid as in the case of the hydrolysis of triglyceride, except in the following system.

$$C \fallingdotseq C_2 < C_3 \ldots\ldots C_8 < C_{10} < C_{12} \fallingdotseq C_{14} \fallingdotseq C_{16} > C_{18}$$

(D). Esters of unsaturated fatty acids are more rapidly attacked than saturated fatty acid esters with the same carbon atoms.

(E). Methyl and ethyl esters of formic, acetic, valeric, benzoic, salicylic and phthalic acid are hardly hydrolysed by pancreas and ricinus lipase.

(F). The differences in the hydrolysis of normalbutyl and isobutyl alcohol esters of fatty acids are not due to the different structures of the alcohols, but to the density of esters. Those between normalpropyl and isopropyl alcohol esters may be, however, attributed entirely to the structure of the alcohols.

Studies on the Absorption Spectra of Wheat Glutenin.

(pp. 159~162)

By Kinsuke Kondo and Hisateru Mitsuta.

(Nutritional Chemical Laboratory, Faculty of Agriculture, and Chemical Institute, Kyoto Imperial University; Received Jan. 12, 1940.)

On the Carbohydrate in Wheat Gliadin.

(pp. 163~174)

By Kinsuke Kondo and Uichiro Sarata.

(Nutritional Chemical Laboratory, Faculty of Agriculture, and Chemical Institute, Kyoto Imperial University; Received Jan. 12, 1940.)

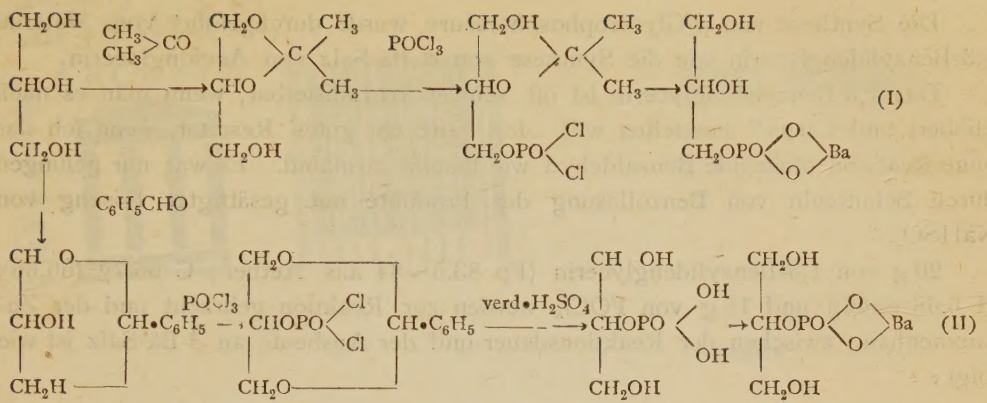
Die Synthese von α- und β- Glycerophosphorsäure.

(SS. 175~180)

Von Yataro Obata.

(Landwirtschaftliches biochemisches Laboratorium der Kaiserlichen Universität zu Tokyo; Eigegangen am 31, Jan. 1940.)

Der Verfasser synthesierte α- (I) und β-Glycerophosphat (II) folgenderweise, um die Trennungsmethode der Isomeren von der Glycerophorsäure nach Karrer und Salomon[1] nachzuprüfen:



Nach Perjodsäureoxydation[2] wurden die Produkte auf Abwesenheit der Acylwanderung[3] geprüft, und mit diesen geprüften Proben wurde ein Versuch über die Entstehung des schwerlöslichen Doppelsalzes mit Ba $(NO_3)_2$ gemacht. Wie schon von Karrer und Benz[4] gezeigt gestaltete das α-Ba-Salz nicht das schwerlösliche Doppelsalz aber trotz des Einspruchs von Kay[5] gestaltete das β-Ba-Salz das schwerlösliche Doppelsalz. Dieses Ergebnis stützt die Methode von Karrer nnd Salomon.[1]

Das α-Isomer, eine Glykolverbindung, ist oxydiert von Pb (IV)-Acetat[6] oder HIO_4.[2] Diese zwei Oxydationsbestimmungen wurden versucht in Vergleichung mit fast gleichem Resultate. Die Oxydation mit Pb (IV)-Acetat bedurfte längerer Zeit (20 Stdn.) und die Reagenz ist unstabil. Im Gegensatz bedurfte die Oxydation mit Perjodsäure nur weniger Minuten (15 Min.) und α-Ba-Salz, ist deshalb viel nützlicher. Die Substanz ist hergestellt vom käuflichen Ca-Salz (Merck) nach der Methode von Karrer und Salomon.[1]

Pb (IV)-Acetat-Oxydation:

Subst. (g)	$Na_2S_2O_3$ (0.097872 N) (cc) gef.	Blindversuch	Oxydationswert (%)
0.1020	8.4	12.7	87.55
0.0465	10.8	"	91.82
0.0629	9.8	"	92.52
0.0443	10.7	"	85.41
0.0587	10.06	"	85.46
0.0459	10.69	"	83.24
			Mittelwert 87.73

HIO_4-Oxydation

	$Na_2S_2O_3$ (0.09834 N) (cc)		
0.0250	11.8	14.7	87.63
0.0250	12.0	14.9	87.63

HIO_4-Oxydation des α-Ba-Salzes, synthetisch hergestellt von Acetonglycerin nach E. Fischer und Pfahler[7]:

Subst.	$Na_2S_2O_3$ (0.09834 N) gef.	Blindversuch	Oxydationswert (%)
0.025 g	11.8 cc	14.8 cc	90.72

Die Synthese von β-Glycerophosphorsäure wurde durchgeführt vom Material 1,3-Benzylidenglycerin wie die Synthese von α-Ba-Salz von Acetonglycerin.

Das 1,3-Benzylidenglycerin ist oft schwer kristallisierbar, wenn man es nach Hibbert und Carter[8] herstellen will. Ich hatte ein gutes Resultat, wenn ich das ohne Reaktion bleibende Benzaldehyd wie Bisulfit ausnahm. Es war mir gelungen durch Schüttenln von Benzollösung der Produkte mit gesättigter Lösung von $NaHSO_3$.

20 g von 1,3-Benzylidenglycerin (Fp 83.5～84 aus Aether; C 66.72 (66.66), H 6.68 (6.66)) und 15 g von $POCl_3$ werden zur Reaktion gebracht und der Zusammenhang zwischen der Reaktionsdauer und der Ausbeute an β-Ba-Salz ist wie folgt:

Reaktionsdauer	Ausbeute	
1 Std.	3.5 g	(10 %)
2	9.5	(28)
4	4.0	(11.7″)
10	2.6	(7.6″)
24	0	(—)

Oxydation von β-Ba-Salz mit HIO_4:

Subst.	$Na_2S_2O_3$(0.09834 N) gef.	Blindversuch	Oxydationswert
0.025 g	14.5 cc	14.6 cc	3.02%
0.025 g	14.6	14.7	3.02

Die Reaktion mit $Ba(NO_3)_2$: 2.5 g der Probe wurden in 50 cc Wasser gelöst, zu 30 cm eingeengt und mit einer Lösung von 2.5 g $Ba(NO_3)_2$ in 50 cm Wasser gemischt.

Bei α-Ba-Salz gab es lange keine Veränderung; bei β-Ba-Saiz gab es sofort eine weisse Trübung und nach 48 Stdn. wurden 2.44 g Präzipität erhalten. Die Ausbeute ist 3.08 g (86%), wenn man 0.64 g (Löslichkeit 0.8%) gelöst zur Lösung hinzufügt. Die Analyse des Produktes: Ba 47.36% (47.04, P 7.12% (7.07).

Bei dem Gemisch von gleichen Mengen von α- und β-Ba-Salzen erreicht die Ausbeute an Doppelsalz 93%.

Diese Untersuchungen wurden von mir unter der Leitung des Herrn Prof. Bunsuke Suzuki ausgeführt, dem ich hiermit für seine Unterstützung meinen besten Dank ausspreche.

(Gelesen in der monatlichen Versammlung der Agrikulturchemischen Gesellschaft, Nov. 1938).

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(Während die Korrektur dieser Abhandlung fand ich den Bericht von Brigl u. Müller (B. **72**, 2126; 1939)).